

ATTACHMENT

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RESEARCH ARTICLE

Cloned transchromosomal calves producing human immunoglobulin

Yoshimi Kuroiwa¹, Poothappillai Kasinathan², Yoon J. Choi³, Rizwan Naeem⁴, Kazuma Tomizuka¹, Eddie J. Sullivan², Jason G. Knott², Anae Dûlêau⁵, Richard A. Goldsby⁵, Barbara A. Osborne⁵, Isao Ishida^{1*}, and James M. Robl^{2*}

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Human polyclonal antibodies (hPABs) are useful therapeutics, but because they are available only from human donors, their supply and application is limited. To address this need, we prepared a human artificial chromosome (HAC) vector containing the entire unrearranged sequences of the human immunoglobulin (hlg) heavy-chain (H) and lambda (λ) light-chain loci. The HAC vector was introduced into bovine primary fetal fibroblasts using a microcell-mediated chromosome transfer (MMCT) approach. Primary selection was carried out, and the cells were used to produce cloned bovine fetuses. Secondary selection was done on the regenerated fetal cell lines, which were then used to produce four healthy transchromosomal (Tc) calves. The HAC was retained at a high rate (78–100% of cells) in calves and the hlg loci underwent rearrangement and expressed diversified transcripts. Human immunoglobulin proteins were detected in the blood of newborn calves. The production of Tc calves is an important step in the development of a system for producing therapeutic hPABs.

Despite the substantial need for hPABs to treat many diseases, the supply is limited to what can be obtained from human donors. Furthermore, the application of hPABs has been restricted because human donors cannot be hyperimmunized, that is, repeatedly boosted with antigen. Transgenic animals carrying hlg loci could provide a source of hPABs, especially targeted hPABs resulting from hyperimmunization with human pathogens or human molecules. Transgenic mice carrying hlg loci have been created^{1–6} and are useful for the derivation of human monoclonal antibody therapeutics^{7,8}.

Transgenic cattle carrying hlg loci could be useful for large-volume commercial production of hPABs. Transgenesis including gene targeting^{9–12} in livestock has been reported; however, the procedures used are not suitable for transfer of the hlg loci¹³ (1–1.5 Mb for each locus) because the maximum size of DNA that can be inserted is very limited (20–100 kb). Mammalian artificial chromosome (MAC) vectors^{14–17} may be a better choice because of their large insert capacity. Thus far, there have been no reports of the transfer of MAC vectors in livestock. Furthermore, human microchromosomes are generally mitotically unstable in a foreign environment^{14,18}. This could be a major obstacle in the production of transchromosomal cattle, which require a large number of cell divisions for full term development.

Another potential limitation of using cattle to produce hPABs is the difference in immunophysiology between cattle and humans^{19–22}. In humans and mice, bone marrow is the major site of origin of all lymphocytes and the location of subsequent B-cell maturation. In contrast, spleen, rather than bone marrow, is the presumed site of B-cell origin and immunoglobulin rearrangement in bovine. Furthermore, because of a limited number of

functional V genes in bovine, gene conversion may be an important mechanism for the generation of diversity, especially for the light chain^{19,20}. Gene conversion occurs in the ileal Peyer's patch, where B cells undergo proliferation and diversification. These differences could impede the functional rearrangement, diversification, and production of hlg in cattle.

In this study, we developed a system for introducing heavy- and light-chain hlg loci into bovine by transferring a 10 Mb HAC vector carrying the loci into primary fibroblast cells and then producing cloned cattle from the Tc cells. We also evaluated the retention of the HAC through early gestation and the functional rearrangement, diversification, and expression of hlg in the blood of Tc calves.

Results

HAC transfer into bovine fetal fibroblasts and nuclear transfer. We constructed two HAC vectors (ΔHAC and ΔΔHAC), each carrying both hlg heavy-chain and λ light-chain loci, using a chromosome-cloning system^{17,23} (Fig. 1). HAC vectors were introduced into bovine primary fetal fibroblasts from CHO clones using an MMCT system (Fig. 2). The life-span limits of bovine primary fibroblast cells required that complete antibiotic selection and DNA-based screening be avoided after MMCT to minimize cell divisions before nuclear transfer. Instead, we picked colonies on the basis of growth and morphology under selection and used them for nuclear transfer as quickly as possible. Nevertheless, the cells were useful only for a few days and could not be cryopreserved. Final selection was done after the rejuvenation and expansion of the cells during the growth of cloned fetuses. At 56–58 days, four

¹Pharmaceutical Research Laboratory, Kirin Brewery Co., Ltd., 3 Miyahara-cho Takasaki-shi Gunma 370-1295, Japan. ²Hematech, LLC, 33 Riverside Avenue, Westport, CT 06880. ³Department of Biology, Amherst College, Amherst, MA 01002. ⁴Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. ⁵Department of Veterinary and Animal Science, University of Massachusetts, Amherst, MA 01003.

*Corresponding authors (jrobl@hematech.com and i-ishida@kirin.co.jp).

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Table 1. Development of cloned embryos derived from Tc fibroblasts

HAC	Nuclear transfer	Blastocysts (%) ^a	Blastocyst transfer	Recipients	Pregnant 40 days (%)	Pregnant 120 days (%)	Offspring (%) ^b
Δ	776	83 (18)	54	32	16 (50)	Fetal recovery	0
ΔΔ	833	122 (21)	56	28	13 (46)	Fetal recovery	1
Δ Regenerate	672	82 (17)	61	37	8 (22)	6 (18)	6 (16)

Fetal recovery was done between 56 and 119 days of gestation. Regenerated cells were produced from Tc fetuses recovered at 56–58 days of gestation.

^aPercentage of blastocysts was calculated as the number of blastocysts per number of fused donor cell oocyte complexes. Average fusion rate in our laboratory was 70%.

^bFor ΔΔHAC nuclear transfers, one fetus was not recovered and developed to term producing a live healthy calf. For ΔHAC regenerated cells, six live calves were born; two from cell line 6032 did not survive past 48 h and the other four remain alive and healthy.

ΔHAC and two ΔΔHAC fetuses were recovered and fibroblast cell lines were regenerated, expanded, and cryopreserved for further analysis and nuclear transfer. Efficiency of development to the blastocyst stage, to 40 days of gestation, and to term is shown in Table 1 for first-generation and re-cloned cells.

Analysis of cloned Tc fetuses. To examine whether the HAC vector was retained through early gestation and whether hlg loci could be functionally rearranged and expressed during early B-cell development in bovine, we analyzed cloned Tc fetuses collected between 56 and 119 days of gestation. Retention of the HACs in the fibroblast lines derived from six cloned Tc fetuses collected at 56–58 days and from an additional seven fetuses collected between 77 and 119 days was evaluated by G418 resistance (Fig. 3A) and by genomic PCR of human *IgH* and *Igλ* loci (Fig. 3B). Of the 13 fetuses, 9 were resistant to G418 (4 ΔHAC and 5 ΔΔHAC) and 8 showed the presence of both human *IgH* and *Igλ* loci. We evaluated five positive 77–119-day fetuses for expression and rearrangement of the hlg loci by reverse transcription-PCR (RT-PCR) analysis, followed by sequencing of the amplified products. Human *IgH* and *Igλ* genes were expressed (Fig. 3C) in all fetuses, predominantly in spleen, consistent with endogenous bovine *Ig*

cell lines (5968, 6032, and 6045) were used for re-cloning. One male calf from cell line 6045 and five female calves from cell lines 5968 and 6032 were produced from 37 recipients (16%, Fig. 4A). Four calves survived and were healthy and phenotypically normal. Retention of the HAC vector was confirmed in all the calves by G418 selection (data not shown), genomic PCR (Fig. 4B), and fluorescent *in situ* hybridization (FISH) analyses (Table 2; Fig. 4C). FISH analysis indicated that the HAC was retained as an independent chromosome and that the proportion of cells retaining the HAC ranged from 78% to 100%. We observed no obvious differences in retention rates between peripheral blood lymphocytes (PBLs; 91%) and fibroblasts (87%). These data demonstrate that somatic-cell re-cloning strategies can be used to produce healthy cloned Tc calves and that the HAC vector can be stably maintained through the large number of cell divisions in bovine development.

Human *Ig* gene expression and protein production in Tc calves. To determine whether hlg loci were rearranged and expressed, we carried out RT-PCR analysis on PBLs. We observed expression of both human *IgH* and *Igλ* genes in the PBLs of all the calves (data not shown). The diversity of the human *IgH* and *Igλ* repertoire was determined by sequence analysis (Table 3). A representative set of the sequences showed a wide utilization of V_H/V_λ, D, and J_H/J_λ segments distributed over the loci. In the *IgH* transcripts, the frequent utilization of J_H4 and of V segments from V_H1 and V_H3 was observed, similar to patterns in human²⁴. Addition of non-germline nucleotides (N addition) and nucleotide deletion were also observed in both hlgH and hlgλ transcripts. These produced a high degree of diversification in the third complementarity-determining region of both the hlgH and hlgλ chains. Furthermore, hlg proteins were secreted at levels ranging from 13 ng/ml to 258 ng/ml (immunoglobulin expression is typically very low to undetectable in newborn calves²⁵) in blood samples collected before colostrum feeding in five of the seven Tc calves. In the two calves in which hlg proteins were not detected, bovine

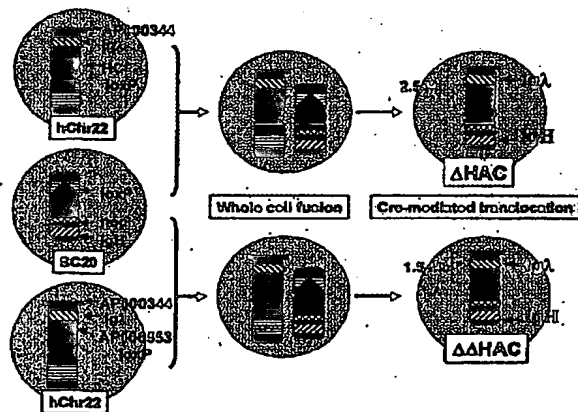


Figure 1. Diagram of ΔHAC and ΔΔHAC construction. In ΔHAC construction, the hChr22 fragment (hChr22), which contains the *Igλ* locus, was truncated at the AP000344 locus and a *loxP* sequence was integrated at the *HCF2* locus. In ΔΔHAC construction, the *loxP* sequence on the hChr22 fragment was integrated at the AP000553 locus. DT40 cells containing the hChr22 fragments were fused with DT40 cells containing the SC20 fragment (SC20 is a fragment of hChr14 and contains the *IgH* locus). Cre-mediated translocation resulted in ΔHAC, which contained a 2.5 Mb hChr22 region, and ΔΔHAC, which contained a 1.5 Mb hChr22 region, each fused to the SC20 vector.

Table 2. HAC retention in cloned Tc calves

Calf number	Cell type	HAC positive/total (%)	Two signals/total (%)
50	PBL	50/50 (100)	6/50 (12)
50	Fibroblast	47/50 (94)	0/50 (0)
1084	PBL	46/50 (92)	0/50 (0)
1084	Fibroblast	34/39 (87)	3/39 (8)
1085	PBL	39/50 (78)	2/50 (4)
1085	Fibroblast	49/60 (82)	0/60 (0)
1086	PBL	47/50 (94)	1/50 (2)
1086	Fibroblast	43/50 (86)	0/50 (0)
Total	Combined	355/399 (89)	12/399 (3)

Retention rate was determined in both PBLs and fibroblasts in each calf by FISH analysis using human Cot-1 DNA as a probe.

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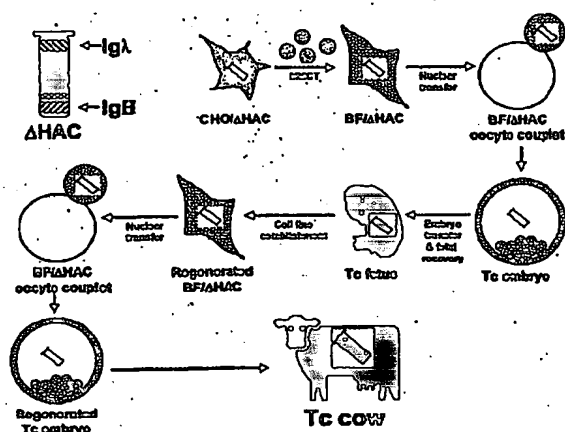


Figure 2. Procedure for production of cloned Tc calves. Structure of Δ HAC (upper left) is shown with hChr22 (green) and hChr14 (red) regions containing *Igλ* and *IgH* loci. The centromere of the HAC is derived from the hChr14 fragment. The HAC was transferred from a CHO clone (CHO/ Δ HAC, light blue) into fetal bovine fibroblasts by means of a MMCT technique. Tc fibroblasts (BF/ Δ HAC, black patterned) and enucleated oocyte (yellow) couplets were fused, resulting in transfer of the fibroblast nucleus and formation of an embryo. The reconstituted Tc embryos were cultured *in vitro* to the blastocyst stage and then implanted into recipient cows. At ~60 days of gestation, Tc fetuses were recovered and Tc fibroblast cell lines were re-established (regenerated BF/ Δ HAC), evaluated, and used for further nuclear transfer. Regenerated Tc embryos were transferred to recipients to produce Tc calves.

immunoglobulin proteins were also not detected. These results demonstrate that the human *IgH* and *Igλ* loci can be functionally rearranged and expressed in cattle.

Discussion

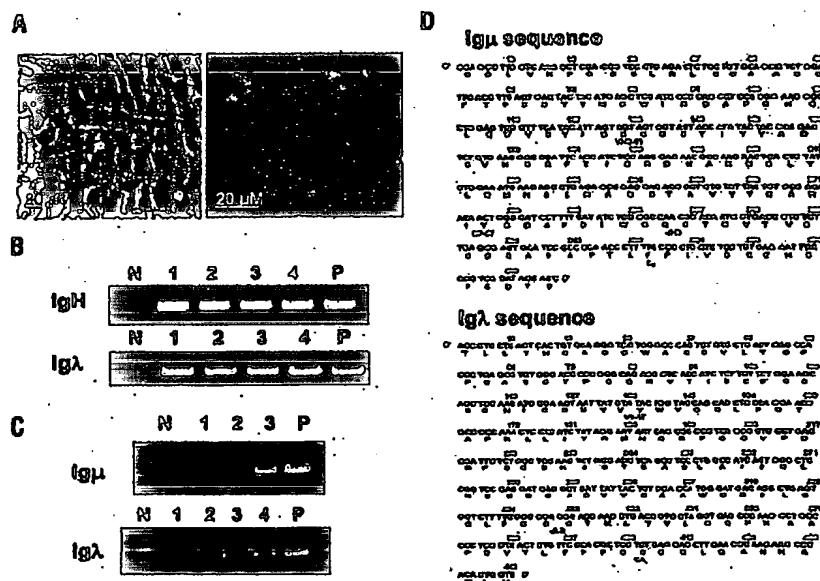
In this study, we demonstrate the production of healthy calves carrying a stable microchromosomal vector encoding human *IgH* and *Igλ* loci, functional diversification of the hIg genes, and production of hIg protein. To accomplish this, several challenges needed to be addressed, including the lack of methods for introducing large foreign DNA sequences into cattle, the possible mitotic instability of a microchromosome vector, and the substantial differences in immune system development between cattle and humans.

MMCT has been accomplished in mice using embryonic stem cells and chimera production followed by germline transmission from embryonic stem cell-derived germ cells¹. Mouse embryonic

stem cells have the advantage of an unlimited life span, rapid growth in culture, a straightforward system for clonal propagation of transgenic colonies, and a high rate of contribution to the germ line in chimeras. Unfortunately, embryonic stem cells with similar properties have not been derived in other species, including cattle²⁶. It was therefore necessary to use a nuclear transfer system with primary somatic cells²⁷ to produce Tc cattle. The limitation of this system is that primary fibroblasts, the cell type of choice, have a life span of ~35 population doublings²⁸. After MMCT, our cells were capable of dividing for only about one week and could not be cryopreserved. Our approach to solving this problem was to carry out incomplete selection after MMCT and complete selection on rejuvenated cloned cell lines, and then produce calves by recloning cell lines. Recloning of transgenic somatic cells and production of offspring has been accomplished in cattle²⁹ with limited success. Our results indicate that rejuvenation of cell lines by recloning is a viable method for the production of large numbers of transgenic animals and the establishment of cryopreserved transgenic cell banks.

In mice, the SC20 HAC vector is retained in 70–80% of fibroblasts¹ and only 30–40% of PBLs (data not shown), revealing limitations in mitotic stability in a foreign environment. Because of the number of cell divisions required for bovine development, the retention rate of the HAC was expected to be low. However, the HAC was retained in all cloned calves at a very high rate, both in

Figure 3. Analysis of Tc fetuses. (A) G418 selection of regenerated Tc fibroblast line (left) and control nontransgenic fibroblasts (right). (B) Genomic PCR of *IgH* and *Igλ* loci in Tc fetuses and controls. The three fetuses, 5968 (lane 1), 6032 (lane 2), and 8045 (lane 3) were derived from Δ HAC fibroblasts; fetus 5580 (lane 4) was derived from Δ HAC fibroblasts. As a control, a nontransgenic fetus (lane N) was recovered and evaluated. Human *IgH* and *Igλ* loci were detected by PCR in all Tc fetuses and in a positive-control human liver DNA sample (lane P), but not in the negative control (lane N). (C) Rearranged and expressed human *Igμ* and *Igλ* transcripts amplified by RT-PCR from negative-control nontransgenic bovine spleen (lane N), from brain (lane 1), liver (lane 2), and spleen (lane 3 and 4) of cloned Tc fetus, and from positive-control human spleen (lane P). (D) A representative nucleotide and deduced amino acid sequence of human *Igμ* and *Igλ* transcripts amplified by RT-PCR from a cloned Tc fetus recovered at 89 days. In the *Igμ* transcript, blue represents the variable region sequence (VH3-11), red represents the diversity region (D7-27), green represents the joining region (JH3) and orange represents the constant region. In the *Igλ* transcript, blue represents the variable region sequence (V1-17), red represents the joining region (JL3), and green the constant region sequence.



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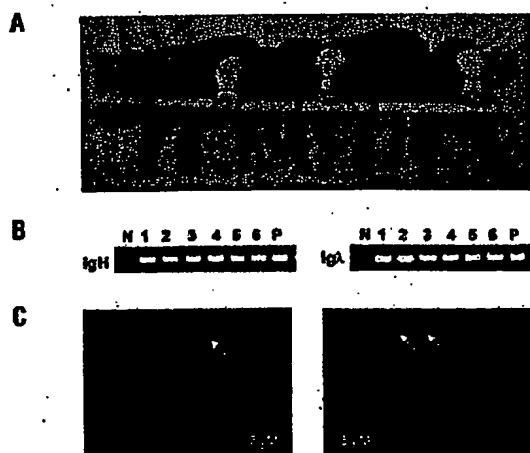


Figure 4. Analysis of cloned Tc calves. (A) Four cloned Tc calves: male calf (50) from cell line 6045 and female calves (1064, 1065, 1066) from cell line 5968. (B) Genomic PCR of *IgH* and *IgA* loci in PBLs from cloned Tc calves and controls: calf 1064 (lane 1), 1065 (lane 2), 1066 (lane 3), 50 (lane 4), 1067 (lane 5), and 1068 (lane 6). Both human *IgH* and *IgA* loci were detected by genomic PCR in all the Tc calves and positive-control human liver DNA (lane P), but not in a negative-control nontransgenic calf (lane N). (C) FISH analysis in metaphase chromosome spreads in a cell showing a single signal and a cell showing a double signal. Arrows indicate location of HACs (red) among surrounding bovine chromosomes (blue). A single HAC per cell is introduced and retained in most cells (left panel); however, improper separation of chromatids at cell division may result in some cells having two microchromosomes (right panel) and some not having a microchromosome.

fibroblasts (87%) and PBLs (91%). In our previous study¹⁷, the mitotically unstable hChr22 fragment was mitotically stabilized when its centromere was exchanged with that of the SC20 vector, suggesting that the mitotic stability of human microchromosomes might be affected by centromeric structure. In this study, the unexpectedly high mitotic stability of the HAC vector in bovine

tromeric structure with factors regulating cell division in cattle as compared with mice. The high rate of HAC retention shows the utility of HAC vectors for stable introduction of foreign genetic elements into the genome of cattle.

We have shown that *hlg* loci can be functionally rearranged and expressed with substantial diversity in cattle despite the differences in immunophysiology between cattle and humans. Both broad usage of VDJ segments and N addition contributed considerably to *hlg* diversity in human *IgH* and *IgA* transcripts in cloned Tc calves. Notably, in bovine *IgA* transcripts, N addition is rarely observed (data not shown). These results indicate that immunoglobulin gene diversification may be more related to genetic sequence than to the environment in which diversification occurs.

Table 3. Repertoire analysis of human immunoglobulin heavy- and λ -chain transcripts in cloned Tc calves

Human μ nucleotide sequences				
VH	N	DH	N	JH
6-1	0	D6-24	3	JH3
TACTGTGCA		AGAGATG	AGA	ATGCTTTTGATGTC
3-33	8	D6-13	3	JH4
ATTACTGTGCGA	AGAACAAA	ATAGCAGCAGCTGGTAC	GAT	CTTTGACTACT
3-15	4	D6-19	4	JH1
ACTGTACCACAGA	TCTG	ATAGCAGTGGCTGGTAC	TGGG	TACTCCAGCA
3-66	2	D2-2	0	JH3
TACTGTGCGAG	TC	GTAGTACCAGCTGCTAT		GATGCTTTTGATGTCT
3-21	6	D2-21	8	JH4
TTACTGTGCGAG	TTTTGG	GTGGTGGT	CACATTIA	GACTACTGGGG
4-39	8	D3-10	3	JH4
ACTGTGCGAGACA	TGAAAAAC	TTCCGGGAGTTAT	AAT	CTACTGGGGCC
1-69	7	D6-13	1	JH4
TTACTGTGCGAG	GGGGATG	GCAGCAGCTGGTAC	C	GACTACTGGGGC
1-8	0	D2-2	12	JH2
ACTGTGCGAGAG		ATTGTAGTAGTACCAGCTGC	CAAGATCGTAAG	TGGTACTTCGAT
1-18	0	D6-24	15	JH4
TTACTGTGC		GAGATGG	GTTTTGTATCCCCAG	TTTGACTACTGG
3-20	4	D7-27	1	JH3
TCACTGTGCGAGAA	TTTT	ACTGGGGA	T	GATGCTTTTGATGTCT
Human λ nucleotide sequences				
VL	N	JL		
1-17	2 (TT)	JL3	TTCCGGCGGAGGG	
2-13	0	JL2	GGTATTCGGCGGAGG	
1-19	0	JL1	TCTTCGGAAGTGGG	
5-2	2 (TA)	JL3	GTTCGGCGGAGAG	
1-7	1 (C)	JL3	TCGGCGGAGGGA	
2-13	0	JL1	TATGCTTCGGAAGTGG	
2-1	0	JL1	TATGCTTCGGAAGTGG	
1-2	1 (G)	JL1	ATGCTTCGGAAGTGG	
1-4	2 (GT)	JL3	TTCCGGCGGAGG	
1-4	0	JL1	GGAAGTGGGA	

Human μ - and λ -specific mRNAs were amplified by RT-PCR, cloned, and sequenced. Nucleotide sequences of VDJ junctions of each of ten independent μ and λ clones are shown, divided into Vh/VL, D, Jh/JL, and N segments, as identified by homology to published germline sequences (Ig-BLAST).

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Several challenges remain before our system can be used for large-scale production of hPABs. Because Tc cattle retain the bovine Ig loci, expression of bovine antibody is expected to dominate over that of human antibody. We have observed this previously in Tc mice in which the murine Ig loci were not inactivated (data not shown). Furthermore, chimeric antibodies containing combinations of human and bovine heavy- and light-chain proteins are expected to be present. Therefore, methods of reducing bovine Ig expression are probably needed before commercial production of hPABs can proceed in Tc cattle.

A Tc bovine-based system for producing therapeutic hPABs would have several advantages: (i) cattle could be hyperimmunized with essentially any human pathogen or human molecule; (ii) cattle produce very large quantities of antibodies; (iii) large numbers of antigens could be evaluated quickly because one line of genetically modified cattle could be used for all antigens; and (iv) scale-up of antibody production would be as straightforward as immunizing additional cows. Therapeutic hPABs produced in a Tc bovine-based system may have broad application in the treatment and prevention of infectious disease (including antibiotic resistant infections), autoimmune disease, and cancer.

Experimental protocol

Construction of HAC vectors. HACs were constructed using a previously described chromosome-cloning system^{17,23} (Fig 1). Briefly, for the construction of ΔHAC, the previously reported hChr22 fragment (hChr22) containing a *loxP* sequence integrated at the *HCF2* locus was truncated at the AP000344 locus by telomere-directed chromosomal truncation. Next, cell hybrids were formed by fusing the DT40 cell clone containing the hCF22 with a DT40 cell clone containing the stable and germline-transmissible human microchromosome vector SC20. The resulting DT40 cell hybrids contained both hChr fragments. The DT40 hybrids were transfected with a Cre recombinase expression vector to induce Cre-*loxP*-mediated chromosomal translocation between hChr22 and the SC20 vector. The stable transfectants were analyzed using nested PCR to confirm the cloning of the 2.5 Mb hChr22 region into the *loxP*-cloning site in the SC20 vector (ΔHAC). ΔHAC was constructed using the same chromosome cloning system except that the *loxP* sequence on hChr22 was integrated into the AP000553 locus, creating a 1.5 Mb insert upon Cre-*loxP*-mediated translocation.

HAC vector transfer into bovine fetal fibroblasts. Bovine fetal fibroblasts were cultured in α-MEM (Life Technologies, Rockville, MD) medium supplemented with 10% (vol/vol) FCS (Life Technologies) at 37°C and 5% CO₂. Microcells were purified from the CHO clone retaining the ΔHAC or ΔΔHAC as described previously¹⁷. Bovine fetal fibroblasts were fused with microcells using polyethylene glycol (PEG 1500, Roche, Nutley, NJ), and the fused cells were selected under 700 μg/ml of G418 (Life Technologies) for 10–14 days. The G418-resistant clones were picked and used for nuclear transfer.

Nuclear transfer. The nuclear transfer procedure was carried out essentially as described previously^{22,23}. *In vitro*-matured oocytes were enucleated

–18–20 h post maturation. Cytoplasm-donor cell couplets were fused using a single electrical pulse of 2.4 kV/cm for 20 μs (Electrocell Manipulator 200, Genetronics, San Diego, CA). At 30 h, post maturation reconstructed oocytes were activated with calcium ionophore (5 μM) for 4 min (Cal Biochem, San Diego, CA) and 10 μg cycloheximide and 2.5 μg cytochalasin D (Sigma) as described earlier²⁴. After activation, cloned embryos were placed in culture in four-well tissue culture plates, containing irradiated mouse fetal fibroblasts and 0.5 ml of ACM culture medium covered with mineral oil (Sigma) and incubated at 38.5°C in a 5% CO₂ in air atmosphere. On day 4, 10% (vol/vol) FCS (Life Technologies) was added to the culture medium. On days 7 and 8, embryos were transferred into synchronized recipients. All animal work was done following a protocol approved by the Trans Ova Genetics (Sioux Center, IA) institutional animal care and use committee.

Genomic PCR analysis. Genomic DNA was extracted from Tc fetuses at 56–119 days of gestation or from cloned newborn calves and subjected to PCR using primers IGHV3 for the human *IgH* locus and IGLC for the human *IgL* locus, as described previously¹⁷.

RT-PCR and repertoire analyses. Total RNA was recovered from spleen, liver, and brain of Tc fetuses or from PBLs of Tc calves. RT-PCR was carried out as described previously²⁵. For human *Ig* transcripts, VH1/5 BACK, VH3 BACK, and VH4BACK were used as a 5' primer and Cμ-2 was used as a 3' primer. For human *Igλ* transcripts, Vλ1LEA1, Vλ2MIX, and Vλ3MIX were used as a 5' primer and CAMIX was used as a 3' primer. The amplified cDNAs were subcloned by using a TA cloning kit (Invitrogen, San Diego, CA) and sequenced using a DNA autosequencer (ABI3700 Sequencer, GlaxoWellcome, Herts, United Kingdom).

FISH analysis. Digital image analysis was done using the Mac Probe system (Applied Imaging, Santa Clara, CA). HAC painting was done using digoxigenin-labeled (Boehringer Ingelheim, Ridgefield, CT) human Cot-1 DNA as a probe, and the digoxigenin signal was detected with an anti-digoxigenin–rhodamine complex that fluoresced red. DAPI (Sigma) was used for background staining. Standard chromosome and FISH protocols were carried out as described²¹.

ELISA analysis for human antibody. Plasma samples were obtained from Tc calves before they were fed colostrum and human Ig levels were determined by solid-phase ELISA. The assay used a bovine anti-human immunoglobulin as the capture antibody and an HRP-labeled sheep anti-human immunoglobulin as the detecting antibody. Amounts of human immunoglobulin >10 ng/ml were reliably detected by this assay.

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Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology website (<http://www.nature.com/naturebiotechnology/>) for details.

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